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Phenotypic Variation in Epitope Expression of the *Neisseria gonorrhoeae* Lipooligosaccharide

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Gonococcal lipooligosaccharides (LOSs) are a series of antigenically complex heteropolymers. To investigate whether all members of clonally selected populations of *Neisseria gonorrhoeae* express antigenically similar LOS, we studied gonococcal strains 4505 and 220 with monoclonal antibodies 6B4 and 3F11 which have specificity for different oligosaccharide epitopes on the same or comigrating LOS unit(s) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fluorescent-antibody and immunoelectron microscopy studies indicated that all members of the clonally selected populations were not homogeneous for the epitopes these antibodies recognized. Fluorescence-activated cell sorting studies of 3F11-coated strain 220 indicated that the density of epitope expression was a function of time of growth. The population could be separated into two broad groups corresponding to organisms staining strongly or weakly for the 3F11 epitope, and the epitope density decreased during the late-log and stationary phases of growth. Sequentially staining organisms on Formvar grids with 6B4 and 3F11, followed by staining with either 5- or 15-nm colloidal gold spheres conjugated to goat anti-mouse immunoglobulin M demonstrated the following populations of cells among organisms derived from a single clone: organisms which stained for both 6B4 and 3F11 epitopes and organisms which stained for either 6B4 epitopes alone or 3F11 epitopes alone. Immunofluorescence microscopy studies with rhodamine and fluorescein goat anti-mouse immunoglobulin M conjugates sequentially staining organisms on Formvar grids with 3F11 and 6B4 also demonstrated these three populations. Analysis of LOS preparations made over the last 5 years indicated no change in serotype antigen concentration or in sodium dodecyl sulfate-polyacrylamide gel electrophoresis migration pattern. These studies indicate that while clonally selected strains of *Neisseria gonorrhoeae* undergo phenotypic variation at the epitope level, the impact of this variation on the total LOS of the population has little overall effect on its antigenic or physicochemical properties.

The lipooligosaccharide (LOS) of *Neisseria gonorrhoeae* plays an important role in the immunity and pathogenesis of gonococcal infection. Studies of the fallopian tube organ culture model indicate that LOS is the principal toxin associated with *N. gonorrhoeae* (7). LOS is important in human immunity since LOS determinants have been shown to serve as targets for human bactericidal antibody (4). Despite the relatively small size of individual LOS units and the absence of repeating O side chains, gonococcal LOS has been demonstrated to have a complex antigenic structure with at least six antigenically distinct serotype determinants (1, 3) and two other LOS antigens which are shared by all (the common determinant) or some (the variable) strains (3). Studies with monoclonal antibody reagents have confirmed this antigenic heterogeneity (2, 17). Recent studies of gonococcal surface protein antigens have focused on the phase variation that these components have been shown to undergo (5, 14, 28, 29). LOS, like its analog in enteric bacteria, lipopolysaccharide, undergoes a complex process of biosynthesis; thus, it is possible that similar disruptions at distinct points in the process could result in phase variation (21). To answer this question, we studied clonally derived gonococci with monoclonal antibodies specific for different LOS epitopes at the organism level to determine whether variation of LOS antigen expression could occur among individual members of that population. The studies presented in this paper indicate that such phenotypic variation in LOS antigen expression does occur but that the effect on the antigenic expression of the entire LOS population cannot be detected with polyclonal antisera or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

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MATERIALS AND METHODS

Bacteria. Strains were obtained from our own collections. Strain 4505 is the prototype strain for LOS serotype Gc 3 (1). Strain WR 220 was obtained from the Walter Reed Collection. Both strains are nonpiliated and opaque. Both strains were maintained in either skim milk or Mueller-Hinton broth containing 10% glycerol at −70°C. All strains were reconstituted on chocolate agar supplemented with IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.).

Monoclonal antibodies. Monoclonal antibodies 3F11 and 6B4 were utilized in this study. Both murine antibodies are immunoglobulin M (IgM) isotype and recognize different epitopes on the same or comigrating LOS units of all wild-type gonococci we have tested (17). Both were developed by standard fusion and screening techniques (2, 13).

Western blot immunoblot analysis. SDS-PAGE was performed by a modification of the method of Laemmli (15) as suggested by Hitchcock and Brown (11). After the dye front migrated to the bottom of the gel, the slab was placed on conditioned nitrocellulose paper and placed in a Transblot (Bio-Rad Laboratories, Richmond, Calif.) apparatus. Transfer of the LOS occurs in 1.5 h at 50 volts (14). After transfer, the nitrocellulose paper was blocked with 3% gelatin, exposed to the monoclonal antibody for 1 h, washed with

TABLE 1. Numerical analysis summary

Time (h)	% of population in specified gates[a]							
	Clone A1		Clone A2		Clone C1		Clone C2	
	10, 60	60, 250	10, 60	60, 250	10, 60	60, 250	10, 60	60, 250
2	20.9	50.4	21.6	45.0	18.1	55.8	14.7	68.8
4	21.2	49.2	18.6	56.2	35.1	26.2	26.4	48.2
6	25.0	42.0	33.0	20.8	35.8	30.3	38.8	36.0
8	29.6	34.0	32.9	30.9	31.2	19.1	38.5	36.2

[a] Clones are all from strain 220. Avidin-fluorescein control, 10, 60 = 56.4%; 60, 250 = 1.65%.

phosphate-buffered saline-Tween, and exposed to ^{125}I-labeled protein A conjugate for 1 h. The paper was washed and exposed to photographic film.

LOS preparations. LOSs were prepared from *N. gonorrhoeae* by the method of Westphal and Jann (31).

Immunoelectron microscopy. Immunoelectron microscopy with goat anti-mouse immunoglobulin bound to gold spheres was the methodology applied to study the phenotypic expression of LOS epitopes (27). Gonococci were grown for 18 h on GC medium base supplemented with IsoVitaleX. These organisms were fixed in 3% glutarldehyde, suspended (approximately 10^6 CFU/ml) in distilled water, spread as a thin film on Formvar-coated nickel grids, and stained with one of the monoclonal antibodies. After incubation, the grids were washed by spraying with distilled water for 15 s, stained with 5-nm anti-mouse IgM colloidal gold spheres (Janssen Biotechnology), incubated, and washed. The second monoclonal antibody was then applied, and the process was repeated with the 15-nm anti-mouse IgM colloidal gold spheres. The sequence of both monoclonal and conjugate application was alternated. This did not affect the results obtained. Experiments were also performed which demonstrated that at the dilutions of conjugate employed, saturation of the monoclonal antibody by the conjugate was complete. These experiments in which each of the monoclonal antibodies was stained successively by the conjugates demonstrated that there was minimal binding of the second conjugate to the monoclonal antibody after binding of the first conjugate. The specimens were then examined with a Siemens Elmskop 1A transmission electron microscope at 80 kV accelerating voltage. Immunoelectron microscopy with ferritin-labeled anti-mouse IgM conjugates was performed on Epon-embedded specimens by the methods described by Pease (23).

Fluorescent-antibody studies. Fluorescent-antibody studies were accomplished by using fluorescein and rhodamine anti-mouse IgM conjugates (Kirkegaarde and Perry). Organisms were placed on glass slides, air dried, dipped in methanol, and stained with either 3F11 or 6B4 followed by either rhodamine or fluorescein conjugate.

FACS analysis. Fluorescence-activated cell sorter (FACS) analysis was utilized to evaluate changes in phenotypic expression with monoclonal antibody 3F11 (30). To ensure clonality of the strain before the experiment, two single colonies of strain 220 were picked and serially passed 11 times. These were designated clones A and C in the presentation of the FACS data. For this technique, 10^7 cells per ml from duplicate cultures of each clone were reacted with biotin-conjugated anti-LOS monoclonal antibody. Control mixtures consisted of organisms without added antibody. The cells were washed and reacted with fluorescein-labeled avidin for 15 min. Cells were rewashed and suspended to a final concentration of approximately 10^6 cells per ml. Using a FACS II flow cytometer equipped with an argon ion laser and interfaced to a Vax computer, we performed dual parameter FACS analyses on these specimens. Cell-associated fluorescein with an excitation of 500 mW at 488 nm and fluorescence detection through a 514-nm narrow-band emission filter were measured. Narrow-angle forward light scatter was measured as the second parameter. All fluorescence and forward scatter measurements were standardized by using fluorescent microspheres of 1.7-μm diameter. Logarithmic amplification of each fluorescence signal was used, and the data, which were obtained by analysis of 10,000 bacteria in each sample, were collected in the list mode. Frequency histograms of fluorescence distribution were generated by plotting the relative number of cells (y axis) versus $\log_{10}$ fluorescence intensity (x axis). The fluorescence distribution of the control mixture was used to establish positive and negative fluorescence gates. The data are presented as the percentage of the population found in each of two gates.

ELISA inhibition analysis. Enzyme-linked immunosorbent assay (ELISA) inhibition studies were performed to determine serotype antigen specificity by the methods of Apicella and Gagliardi (3).

RESULTS

To study the stability of phenotypic expression of LOS epitopes during in vitro growth, we did the following experiments. The phenotypic expression of LOS epitopes recognized by monoclonal antibodies 3F11 and 6B4 on individual gonococci was studied. Studies of ferritin staining of 3F11-treated strain 4505 grown for 2 h in Mueller-Hinton broth indicated that while the majority of the organisms in the population stained, examination of multiple fields from multiple sections indicated that there were organisms present which failed to stain with this antibody. Similar studies of organisms grown in supplemented GC broth at 4, 6, and 8 h showed a progressive decrease in members of the population which stained for the 3F11 epitope (data not shown).

FACS analysis. To determine the dynamics of 3F11 epitope expression over time in more quantitative experiments, we performed studies utilizing FACS analysis. The results with two clonal populations of strain 220 are seen in Table 1. In this table, the 10, 60 gate represents the organisms not stained or weakly staining with 3F11, while the 60, 250 gate represents organisms staining strongly with 3F11. In all instances, the percentage of the population in specified gates shifted from predominately stained (45 to 68.8%) at 2 h to equal numbers of stained and unstained at 8 h. These data indicate that there was a highly reproducible reduction in fluorescence (3F11) binding in the entire population as growth progresses from early to mid-log phase (2 h) to stationary phase (8 h). Growth curves were measured by A_{660} and indicated that slow growth was still occurring at 8 h when the organisms were entering the stationary phase. Therefore, autolysis of organisms did not appear to contribute to these results. Figure 1 shows a composite of FACS studies of one clone of strain 220 at 2, 4, 6, and 8 h. The shift from predominately 3F11 stained at 2 h growth to unstained or less well stained cells at 8 h can be seen.

Immunoelectron microscopy. Further evidence of the scope of this phenotypic variation in LOS epitope expression was obtained from the experiments described below. In these experiments, organisms were stained with 6B4 and 3F11 and counterstained and examined in such a way that binding of both antibodies could be evaluated on individual

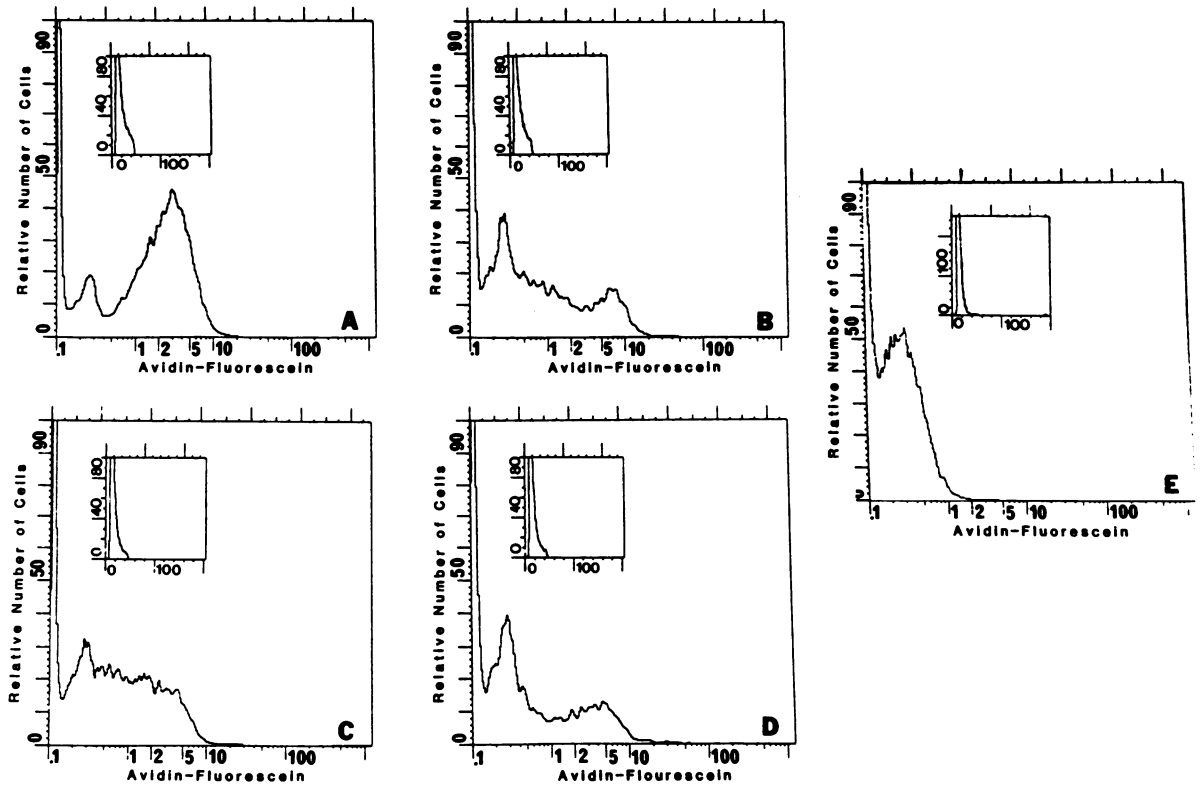

FIG. 1. FACS analysis of clone C1 of strain 220 grown for 2 (A), 4 (B), 6 (C), and 8 (D) h. Biotinylated monoclonal antibody 3F11 followed by avidin-fluorescein was used to stain the organismns. Panel E depicts the results obtained with the avidin-fluorescein alone. Forward scatter for each experiment is presented in the insert in each panel.

members of the clonally selected populations simultaneously. In these experiments, gonococci selected from single colonies of strains 4505 and 220 were grown for either 2 h in supplemented GC broth or 18 h on chocolate agar. Broth-grown organisms were studied directly on Formvar grids, while single colonies of organisms grown on agar were selected and suspended in phosphate-buffered saline, placed on Formvar-coated nickel grids, and stained with one monoclonal antibody (3F11 or 6B4) followed by anti-mouse IgM bound to 15-nm colloidal gold spheres. The grid was then stained with the second monoclonal antibody (3F11 or 6B4), followed by anti-mouse IgM bound to 5-nm colloidal gold spheres. The grids were then examined at ×20,000 with a transmission electron microscope. Figures 2, 3, and 4 show the results of these studies. After 18 h of growth on solid medium or 2 h of growth in broth, individual progeny from clonally selected populations were present which expressed different LOS epitopes. Figure 2A shows two strain 4505 organisms obtained from a single colony after 18 h of growth on chocolate agar exhibiting different LOS epitope patterns in the same electron microscope field. One organism (Fig. 2B) stains with both 15-nm gold spheres (6B4 epitope) and 5-nm gold spheres (3F11), while the second organism (Fig. 2C) stains only with 5-nm gold spheres (3F11). Studies performed with strain 220 under the same growth conditions gave similar results. In addition, members of a colony of strain 220 grown to the mid-log phase (2 h) in supplemented GC broth were studied. Members of this population also demonstrated these phenotypic differences in LOS epitope expression (Fig. 3 and 4). A variety of combinations of staining was seen in these experiments. Over 90% of the populations studied in the mid-log or stationary phase stained for both LOS epitopes. Rare organisms (Fig. 4) were present which did not stain for either epitope. The remainder stained for either the 6B4 or 3F11 epitope.

Fluorescent-antibody studies. Similar results were obtained with rhodamine and fluorescein goat anti-mouse IgM conjugates staining strain 220. Analysis of identical fields of organisms derived from a single 18-h-old colony of strain 220 stained with 3F11 and counterstained with a goat anti-mouse IgM–fluorescein conjugate followed by staining with 6B4 counterstained with a goat anti-mouse IgM–rhodamine conjugate demonstrated that multiple organisms are present in the many fields which stain for both epitopes. In addition, organisms were clearly visible which stained for 6B4 or 3F11 alone (data not shown).

ELISA inhibition and SDS-PAGE. To determine the effect of this variation in epitope expression on the LOS serotype antigen determinant, three LOS preparations which were prepared at the time of this study and 2 years and 5 years before, respectively, were analyzed in a serotype Gc 3-specific ELISA inhibition assay and by SDS-PAGE. The ELISA inhibition analysis for serotype Gc 3 LOS antigen indicated that the three preparations had 90% inhibition values of 10, 5, and 5 μg/ml, respectively, and that all had similar 50% inhibition values (approximately 1 μg/ml). The M_rs of the strain 4505 band in SDS-PAGE of the three LOS preparations were identical (data not shown). This would indicate that the phenotypic variation which was occurring within the members of the 4505 population was not having a detectable effect on the gross physicochemical or antigenic expression of the population.

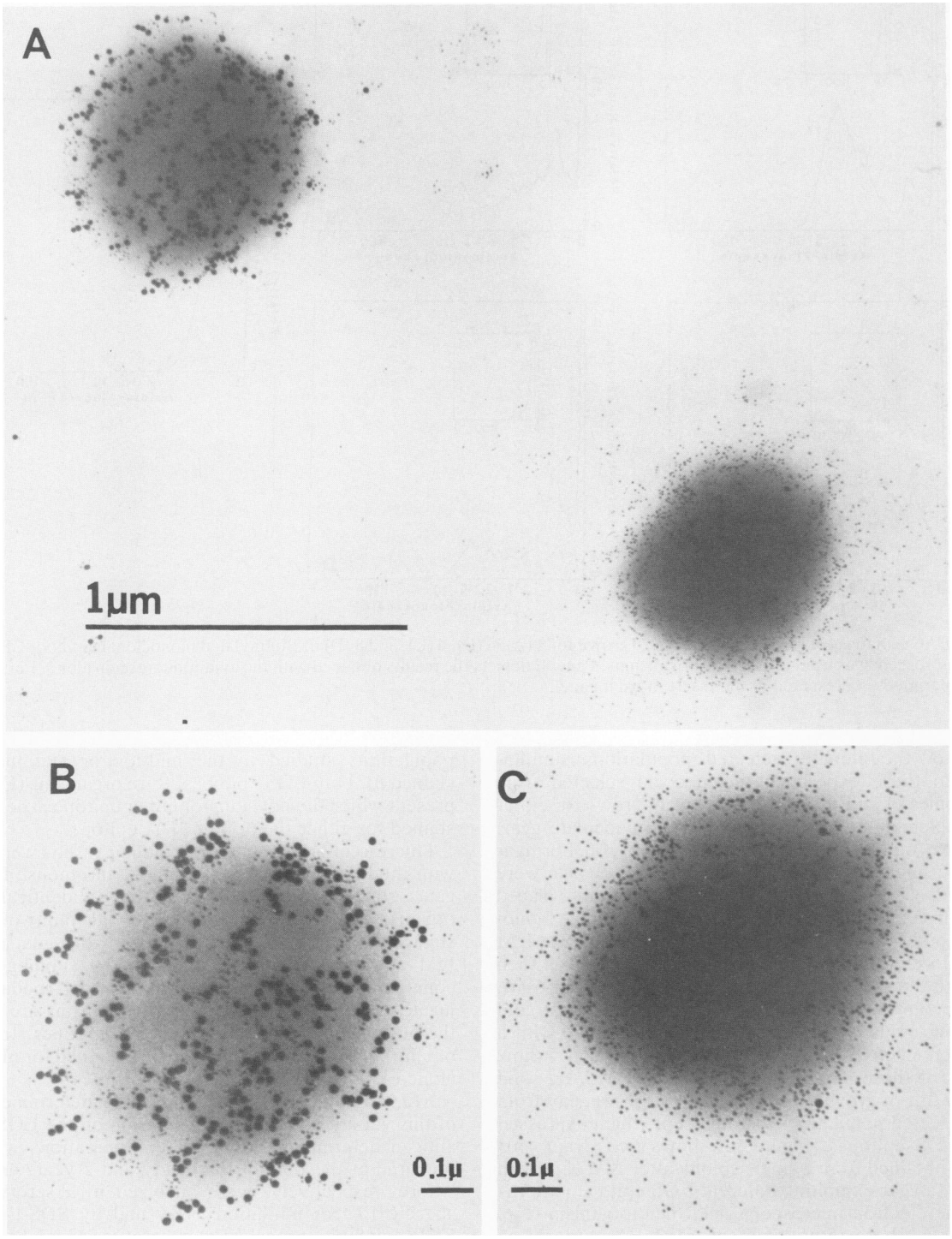

FIG. 2. (A) Immunoelectron micrograph demonstrating differences in phenotypic expression of the 3F11 and 6B4 LOS epitopes on organisms grown for 18 h from a single colony of *N. gonorrhoeae* 4505. In this panel, organisms on Formvar grids were treated with 6B4 antibody followed by goat anti-mouse IgM conjugated to 15-nm gold spheres. The grid was then stained with 3F11 followed by goat anti-mouse IgM conjugated to 5-nm gold spheres. As can be seen, one organism stains for both 3F11 and 6B4, while the second stains only for 3F11. Magnification, ×50,000. (B) Immunoelectron micrograph demonstrating the organism in panel A which contains both 3F11 (5-nm gold sphere) and 6B4 (15-nm gold sphere) LOS epitopes. Magnification, ×80,000. (C) Immunoelectron micrograph demonstrating organism in panel A which contains the 3F11 (5-nm gold sphere) LOS and lacks the 6B4 LOS epitope. Magnification, ×80,000.

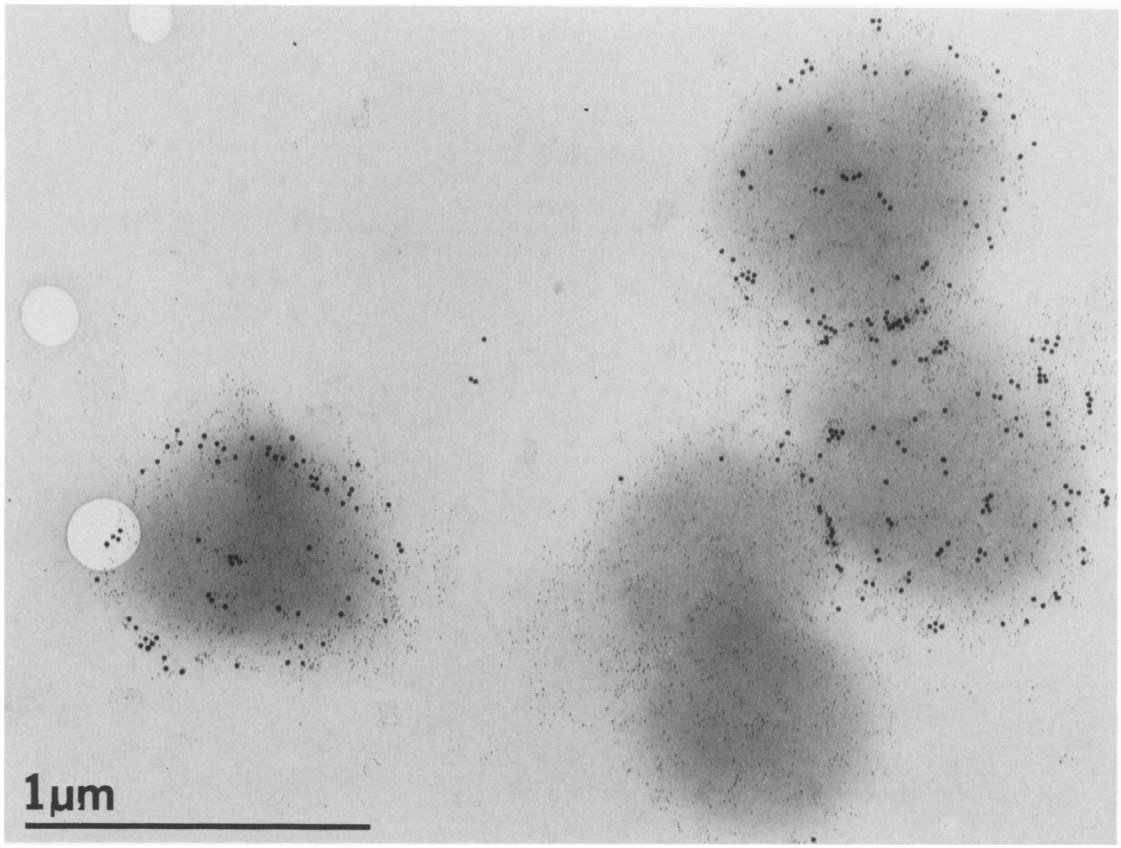

FIG. 3. Immunoelectron micrograph of clonally selected strain 220 grown for 2 h in broth, using colloidal gold staining for the detection of 3F11 (15 nm) and 6B4 (5 nm). As can be seen, three organisms are present, two of which stain for both 3F11 and 6B4, while the remaining organism stains only for 6B4. Magnification, ×45,000.

DISCUSSION

Previous studies with continuous culture techniques have demonstrated the effect of the nutritional environment on gonococcal LOS antigen expression (20). The present studies extended this observation by using FACS analysis of specific LOS epitopes during growth in broth cultures. In addition, using two monoclonal antibodies with specificity for different epitopes on the oligosaccharide units of gonococcal LOS, we demonstrated that phenotypic variation of LOS epitope expression is occurring during in vitro growth among organisms derived from single colonies obtained from two different gonococcal strains. Colloidal gold studies indicated that the 6B4 and 3F11 epitopes are located on distinct LOS chains since geographically discrete staining for both epitopes can be recognized on individual organisms. Thus, in addition to indicating that phenotypic differences in LOS structure can exist between members of a clone, these studies demonstrate that LOS chains with different antigenic composition can exist on the same organism.

Despite the variation at the epitope level demonstrated in this study, the gross antigenic structure of LOS as defined by absorbed polyclonal antisera has been demonstrated to remain constant in different LOS preparations isolated over a 5-year period of study. Given this constancy of the antigenic expression of individual LOSs and of the reproducibility of the LOS banding pattern of an individual strain by SDS-PAGE analysis, the regulation of the epitope variation described in this paper must be controlled within narrow limits by either environmental or genetic factors or a combination of both.

The biosynthesis of enteric lipopolysaccharide has been shown to involve a series of discrete steps which include (i) the synthesis of the lipid A (9, 24, 25), (ii) the synthesis of the core oligosaccharide (6, 8, 10, 16), (iii) the assembly of the O side chain polysaccharide on an undecaprenol carrier (21, 22, 26), (iv) the polymerization of the repeating units on the carrier lipid (22), (v) the translocation of the side chain polysaccharide onto the core (22), and (vi) the postassembly modifications which often involve the addition of glucosyl and other branch structures to certain positions of the side chain (22, 32). Despite the conserved nature of the epitopes recognized by 3F11 and 6B4, they are in some way associated with the serotype determinants of gonococcal LOS because loss of the serotype determinants in pyocin-selected mutants (2, 19) has been shown to be associated with concomitant loss in the expression of the 3F11 and 6B4 epitopes on the intact LOS (2, 17). If biosynthetic mechanisms analogous to those given above can be proposed for gonococcal LOS, it would appear that it is most likely that alterations in some process involving either the translocation of side chains onto a core structure or postassembly modification of branch structures on the side chains are responsible for the antigenic variation seen with these two monoclonal antibodies. Such processes could be controlled by single-enzyme processes and would be subject to relatively minor genetic variation for differences in expression.

The phase variation of outer membrane protein II and

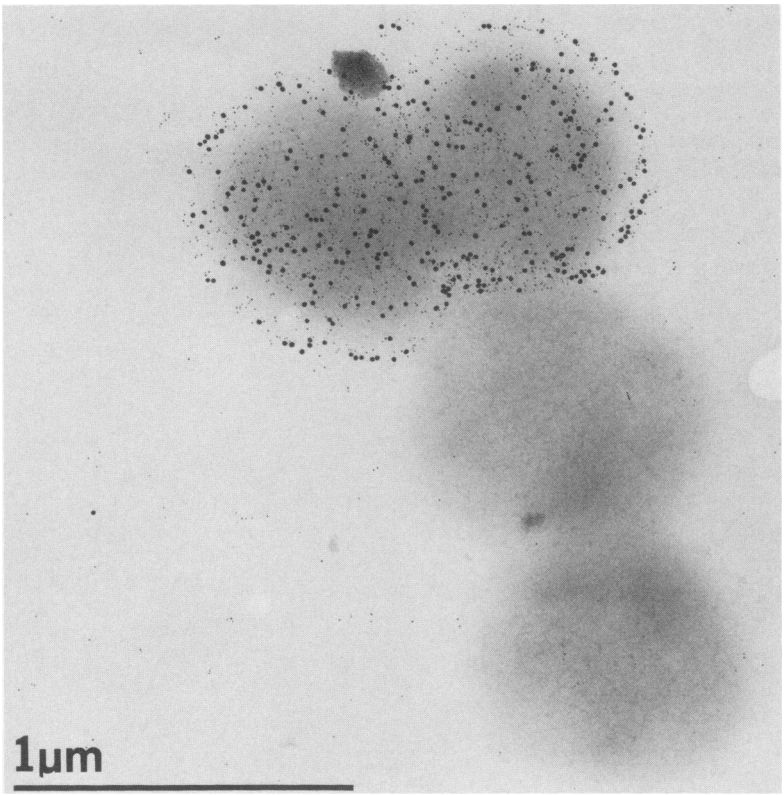

FIG. 4. Immunoelectron micrograph of clonally selected strain 220 grown for 2 h in broth, using colloidal gold staining for the detection of 6B4 (15 nm) and 3F11 (5 nm). One organism stains for both epitopes, while the second stains minimally for 3F11. Magnification, ×45,000.

pilus have been well described (5, 28, 29). It has been shown that during phase variation, the pilin gene is turned on and off at high frequencies (28). Two loci on the gonococcal chromosome function as expression sites for the pilin gene, and many other sites contain silent, variant pilin sequences. When gonococcal cells switch from the pilus-expressing state to the nonexpressing state, genome rearrangement occurs. This leads to the considerable antigenic variation in pilin which can occur in a single strain. It is unlikely that the LOS variation has as its cause a similar genetic basis. The total antigenic expression of the LOS is not modified significantly by the variation at the epitope level, and recent studies would indicate that transformation to cells with expression of both epitopes is constantly occurring (C. A. Hammack, J. M. Griffiss, M. A. Apicella, and H. Schneider, *in Proceedings of the 5th International Conference on Pathogenic Neisseria*, in press).

We are attempting to determine whether this variation also occurs in vivo. Studies of genital secretions from infected humans indicate that both the 3F11 and 6B4 epitopes are present on the same gonococci in these secretions. We are not able to determine now whether organisms are undergoing the in vitro antigenic variation we described in this paper. Recently, Hammack and co-workers using the same monoclonal antibodies described in this manuscript isolated colonies from clonal populations which have different expressions of the 6B4 and 3F11 epitopes on the LOS of the organisms in their population (Hammack et al., in press).

These studies indicate that phenotypic variation of gonococcal LOS occurs under conditions of in vitro growth. Recent advances in molecular biology have led to the isolation of the biosynthetic genes for the lipopolysaccharide of *Salmonella typhimurium* (12) and *Vibrio cholerae* (18). The gene order for *S. typhimurium* lipopolysaccharide has been determined (12). A recent report has indicated that the biosynthetic genes for gonococcal LOS have been expressed in *Escherichia coli* (D. A. Palermo and V. L. Clark, *in Proceedings of the 5th International Conference on Pathogenic Neisseria*, in press). Expansion of these studies will lead to a definition of the mechanisms responsible for the phenotypic variation of LOS epitope expression seen in this study. Further studies are necessary to define the mechanisms responsible for this observation.

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